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Cover Letter

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Dr. Abel Lajtha
Editor in Chief

Neurochemical Research

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Dear Dr. Lajtha:

Please find attached the original manuscript entitled: "*Insulin action on polyunsaturated phosphatidic acid formation in rat brain. An "in vitro" model with synaptic endings from cerebral cortex and hippocampus"* by Sandra E. Zulian, Monica G. Ilincheta de Boschero and Norma M. Giusto, which we would like you to consider for publication in *Neurochemical Research.*

Looking forward to your reply, I remain, yours sincerely,

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Insulin action on polyunsaturated phosphatidic acid formation in rat brain. An "in vitro" model with synaptic endings from cerebral cortex and hippocampus

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Running title: Insulin and diacylglycerol kinase activity in synaptic terminals.

Abstract

The highly efficient formation of phosphatidic acid from exogenous 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) in rat brain synaptic nerve endings (synaptosomes) from cerebral cortex and hippocampus is reported. Phosphatidic acid synthesized from SAG or 1,2-dipalmitoyl-sn-glycerol (DPG) was 17.5 or 2.5 times higher, respectively, than from endogenous synaptosomal diacylglycerides. Insulin increased diacylglycerol kinase (DAGK) action on endogenous substrate in synaptic terminals from hippocampus and cerebral cortex by 199% and 97% respectively. Insulin preferentially increased SAG phosphorylation from hippocampal membranes. In CC synaptosomes insulin increased phosphatidic acid (PA) synthesis from SAG by 100% with respect to controls. Genistein (a tyrosine kinase inhibitor) inhibited this stimulatory insulin effect. Okadaic acid or cyclosporine, used as Ser/Threo protein phosphatase inhibitors, failed to increase insulin effect on PA formation. GTPγS and particularly NaF were potent stimulators of PA formation from polyunsaturated diacylglycerol but failed to increase this phosphorylation when added after 5 min of insulin exposure. GTPγS and NaF increased phosphatidylinositol 4,5 bisphosphate (PIP2) labeling with respect to controls when SAG was present. On the contrary, they decreased polyphosphoinositide labeling with respect to controls in the presence of DPG. Our results indicate that a DAGK type 4 (DAGKε) which preferentially, but not selectively, utilizes 1-acyl-2-arachidonoyl-sn-glycerol and which could be associated with polyphosphoinositide resynthesis, participates in synaptic insulin signaling. GTPγS and NaF appear to be G protein activators related to insulin and the insulin receptor, both affecting the signaling mechanism that augments phosphatidic acid formation.

Key words: phosphatidic acid, diacylglycerol, insulin, synaptosomes

Introduction

Brain insulin receptors (IRs) are present in particularly high concentrations in neurons and abundant IRs are found in cell bodies and synapses (1). Several studies have drawn links between insulin signaling and intracellular trafficking and plasma membrane expression of ion channels and neurotransmitter receptors at the central nervous system, mainly cerebral cortex (CC) and hippocampus (Hp) synapses (2; 3)..

It is known that growth factor transduction acts partly by PA generation through phospholipase D (PLD) activation. We previously explored insulin action on different enzymes in relation to PA and DAG generation. It has recently been reported that after insulin action, PA formation is stimulated in CC synaptosomes from adult rats (4) and that PLD and diacylglycerol kinase (DAGK) activities were strongly stimulated by insulin. Phospholipase inhibitors were then used to identify the role of two phospholipases in DAGK activation by insulin. Our results indicate that DAGK acts in response to increased DAG generated by PIP2-PLC and PLD-PAP2 pathways (5), this action being reinforced by an additional DAGK activation mechanism exerted by the hormone. Insulin induces significant DAGK stimulation in the presence of exogenous [3H] DAG, which exhibits a complex fatty acid composition. Our results also show that in CC synaptosomes, insulin preferentially stimulates PA formation through SAG as exogenous substrate (5).

The arachidonate-enriched DAG pool that arises during stimulus-induced PI turnover is rapidly phosphorylated by a DAGK, much of this PA ultimately converting back into PI (4; 6; 7). Other DAG species are phosphorylated much more slowly. Since of the ten DAGK isoforms only DAGKε shows substrate selectivity (8) and other DAG kinases show no fatty acyl selectivity, it is likely

that arachidonoyl-DAG kinase is the isoform responsible for this DAG phosphorylation. Regulation of arachidonoyl-DAG kinase by PIP2 feedback has been demonstrated in vitro showing PIP2 to be a potent and specific inhibitor of this isoform (9). The stimulation of other DAG kinases by PIP2 and PA, while raising the possibility that these enzymes may be activated by PA or polyphosphoinositides (PPI), does not support their role in regulating the level of PIP2. Given the marked enrichment of animal cell phosphatidylinositols in arachidonate at the glycerol sn-2 position, the substrate selectivity of arachidonoyl-DAG kinase is indicative of a special role of DAGKε in PI resynthesis.

Here we carried out studies on CC synaptic endings to evaluate potential regulators of insulin action on PA synthesis from exogenous saturated (DPG) and unsaturated (SAG) diacylglycerol. Our experimental model represented conditions where DAG from PC-PLD or from PIP2-PLC is present. Insulin action on PA formation could therefore be studied independently of its action on DAG production. Insulin action on DAGK activity in synaptic endings from rat Hp was also explored.

To characterize insulin action on CC synaptosomal DAGK, we analyzed PA formation in the presence of either Ser/Thr and Tyr protein phosphatase inhibitors or a Tyr kinase inhibitor. Type I DAGK isoforms (α, β and γ) are mainly present in CNS, R59022 and R59949, being potent inhibitors of these calcium dependent isoforms (10). To evaluate the possible involvement of type I DAGK action on exogenous DAG, we used these inhibitors in assays with endogenous or exogenous lipid substrate. Since PIP2 is a potent inhibitor of DAGKε, GTPγS was used as G protein activator to increase PIP2 hydrolysis. NaF was also evaluated as a potential regulator of insulin action on DAGK activity.

Our results suggest that DAGKε could participate in insulin signaling in CC synaptosomes and that activation of this enzyme is mediated through the regulation of PIP2 levels. NaF and GTPγS appear to be G protein activators that activate PA formation in IR-related transduction pathways.

Experimental Procedures

Materials

Four-month-old (adult) Wistar-strain rats were used. Animals were kept under constant environmental conditions and fed on a standard pellet diet. They were killed by decapitation and cerebral cortices and hippocampus were immediately dissected from rat brain hemispheres (2-4 min after decapitation). Synaptosomal fraction was obtained following Cotman (1974) with slight modifications (11). [γ-32P]ATP (3,000 Ci/mmol) and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA). All the other chemicals were from Sigma Aldrich (St. Luis, MO).

Determination of DAGK activity

DAGK activity was determined in purified synaptosomal membranes measuring radioactive phosphate incorporation into PA using [γ-32P] ATP and a) endogenous DAG as substrate or b) different concentrations of DPG or SAG (specified in the text figures) as exogenous substrates. The standard assay contained 0.1 % DMSO or 50 mM octyl-β-glucopyranoside (OG), 50 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM DTT and 0.5 mM ATP in a volume of 200 μl. When exogenous DAG was added to the assay, an appropriate volume of DAG stock solution was evaporated under a stream of nitrogen in a glass test tube. It was then re-suspended in DMSO or in OG and the detergent-lipid mixture was sonicated in a test tube into a Cup Horn system (Branson Digital Sonifier,

model 450). Prior to incubation, synaptosomes were also sonicated during 15 sec with radioactive ATP and detergent-lipid suspension in a water bath (Branson Sonifier). Reactions were performed at 37 ºC during 5 min. Integrity of incubated synaptosomes in 0.1 % DMSO (endogenous DAG condition) or with DMSO-DPG or DMSO-SAG conditions was controlled by fluorescence mycroscopy with Nile Red stain.

Blanks were prepared identically, except that membrane fractions were boiled for 5 min before use.

For a standard assay, when [γ-32P] ATP was used as radioactive substrate, reactions were stopped by adding chloroform/methanol/1N HCl (2:1:0.2, by volume) and lipids were extracted following Folch et al. (1957). Five additional washes of the lipid extract for [γ-32P] ATP elimination were carried out using theoretical upper phase. PA, PIP and PIP2 were separated by TLC on 1% potassium oxalate in silica gel H developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol) (12). Lipids were visualized by exposure of the chromatograms to iodine vapors and scraped off for counting by liquid scintillation spectroscopy. Protein was determined according to Bradford's method (1976).

Statistical analysis

All data are given as means ± SD. Statistical analysis was evaluated by the Student´s t-test or one-way analysis of variance (ANOVA) followed by Tukey´s post hoc test. Statistical significance was set at $P<0.05$.

Results

Exogenous SAG was preferentially used for PA synthesis in CC synaptosomes

To evaluate PA formation in synaptosomes from CC, exogenous DAG, DPG and SAG were presented in a micellar assay with 50 mM OG. PA formation from SAG was 17 times higher than with endogenous DAG under this condition, and PA formation from DPG 2.4 times higher than with endogenous DAG (Table I). As previously reported the OG assay appears to be ideal for evaluating DAGK action on SAG (6). The preferential SAG over DPG utilization was also observed in the DMSO assay (Table I). It is interesting to note that PIP and PIP2 labeling was negligible under OG condition (data not shown).

The preferential transformation with 250 μM SAG also occurs when the enzyme action on DPG is at saturating concentration. In order to study kinetic properties we compared synaptosomal DAGK action on exogenous DAG using different ATP and DAG concentrations. PA labeling with DPG as substrate increased as a function of increasing ATP concentrations, reaching saturation at 2 mol% DAG and 4 mM ATP. PA formation from SAG reached saturation at 4 mol% SAG and 4 mM ATP (data not shown). Since our experiments were carried out at 0.5 mol% DAG and 0.5 mM ATP, the saturating concentrations for DPG and ATP are not a factor to be taken into account. The results of the OG micellar assay indicate that exogenous SAG was preferentially used for PA synthesis in CC synaptosomes.

SAG is not a positive regulator for non-acyl selective DAG kinases

It was initially believed that preferential utilization of SAG for PA formation was only apparent and that unsaturated DAG could act as a positive regulator for non-selective DAGK isoforms. To explore this possibility, synthesis of PA from 10 μM SAG was measured in the absence and presence of 250 μM DPG. Controls were carried out with 10 μM and 250 μM DPG only. As shown in Figure 1, PA formation with 250 μM DPG was three times higher than with 10 μM DPG (160 and 40 pmole/mg prot x 5 min respectively). Interestingly, in the

presence of only 10 μM SAG, PA formation was 190 pmole/mg prot x 5 min PA formation when 10 μM SAG was added to 250 μM DPG was only additively increased (376 pmole/mg prot x 5 min). These results suggest that: 1) SAG seems not to be a positive regulator for non-acyl selective DAG kinases; and 2) under the present assay conditions only enzymatic activity from one isoform (DAGKε) was detected as having different kinetic parameters for saturated and unsaturated DAG or, alternatively, different isoform activities were detected, among them DAGKε.

Neither R59022 nor R59949 inhibited PA synthesized from DPG or SAG

R59949 and R59022 (10) inhibit DAG phosphorylation by erythrocyte ghosts and platelet membranes, and also inhibit some DAGK isozymes. It was recently reported that R59949 action on DAGK isoforms is selective, all calcium-activated DAGK members being sensitive to this agent whereas Ca++-insensitive isoforms were only slightly affected. A marked reinforcement of R59949 DAGK inhibition by MgATP (up to 0.2 mM) was also reported. Neither R59022 nor R59949 inhibited arachidonoyl-DAG-specific DAGK from testis (10).

Assays with 250 μM DPG or SAG in the presence of 10 μM of R59022 (at 0.5 mM ATP) and 0.24 μM of R59949 (at 4 mM ATP) were carried out. As previously shown, preferential unsaturated DAG utilization was optimal in OG assays (Table I). Since it has been reported that R59022 and R59949 are poorly soluble in OG, which is commonly used to disperse the DAG substrate (10), a DMSO assay was performed. Neither R59022 nor R59949 (at 0.5 or 4 mM ATP respectively) inhibited PA synthesized from DPG or SAG in the absence of Ca++ (Fig. 2, upper panel, white bars). A similar result was obtained when Ca++ was included with exogenous DAG in the assay (Fig. 2, lower panel). However, R59022 is shown to inhibit DAGK activity using

endogenous DAG as lipid substrate in the presence of Ca++. It has been reported that in particulate fractions of vascular tissue, using micelar SAG in 73 mM OG (13), R59022 has inhibitory effects on DAGK, even at 3 μM, thus indicating that this inhibitory concentration is sufficient when the enzyme is sensitive.

The preferential use of SAG over DPG for PA synthesis could be due to the different kinetic parameters of DAGK activity for saturated and unsaturated DAG substrates detected in our assay. Summing up, this enzyme activity 1) is activated by OG; 2) is insensitive to R59022 and R59949 in the absence of calcium and 3) is insensitive to R59022 in the presence of calcium.

Insulin stimulated DAGK activity through a tyrosine kinase-dependent mechanism

As showed in Fig 2, upper panel, (controls) insulin increased PA formation from DPG and SAG to the same extent. Inhibitors were not able to modify this increased PA labeling in the presence of insulin.

Different potential regulators were assessed in order to find out how insulin affects DAGK action on SAG. The tyrosine kinase activity of the insulin receptor appears to be essential for certain cellular responses to insulin. The tyrosine phosphotransferase function of the insulin receptor is an absolute requirement for the hormone to activate the receptor signaling function in cells. Genistein, a tyrosine kinase inhibitor and vanadate, a tyrosine phosphatase inhibitor, were then used to indirectly assess IR mediation in DAGK activation. It is interesting to note that DAGK assays, as with other phosphorylation assays, usually contain 10–20 mM NaF as protein phosphatase inhibitor. However, it was reported that solubilized insulin receptor and IGF-I receptor preparations in the presence of NaF caused an almost total inhibition of receptor autophosphorylation and tyrosine kinase activity toward exogenous substrates (14). It was also observed that the inhibitory effect of fluoride on

phosphotransferase activity was not due to the formation of aluminum complexes and was dependent on the Mg2+ concentration in the medium (15). Experimental conditions were then modified to evaluate insulin action on PA formation in the absence and presence of NaF.

As shown in Figure 3, insulin in the presence of vanadate (I+V) gave rise to a one-fold increase in DAGK action on SAG. When genistein plus insulin were present, PA synthesis was the same as in the controls. NaF plus I+V stimulated a one-fold increase in PA formation with respect to I+V alone.

Figure 4 shows comparative data from SAG and DPG phosphorylation in the absence and presence of insulin. Data were calculated as percentages of DAGK activity on endogenous DAG in the basal condition. PA formation from SAG is higher than from DPG, even in the presence of insulin. As a consequence of the high preferential utilization of SAG, PA formation increased nine fold with respect to endogenous values in the presence of insulin. Figure 4 (insert), where data are expressed as percentage with respect to incorporation values of insulin condition, shows how NaF reinforces (0.5 fold) insulin's stimulatory effect on PA formation from saturated and unsaturated DAG to a similar degree. However, in the presence of insulin + NaF, PA formation from SAG is 16 times higher than from endogenous DAG and PA formation from DPG is only 8 times higher.

The stimulatory effect of NaF on PA synthesis is not related to Ser/Thr protein phosphorylation protection

As mentioned, NaF is a classical Ser/Thr protein phosphatase inhibitor (16) and is routinely included in extraction buffers to prevent dephosphorylation of proteins on Ser and Thr residues by endogenous phosphatases. We therefore examined the effect of different inhibitors of Ser/Thr protein phosphatase activities on PA synthesis using 250 μM SAG and DPG as substrate (Figure 5). Okadaic acid (a PP1 and PP2A inhibitor) and cyclosporin

A (PP2B inhibitor) were assessed at low (0.5 mM) ATP concentration. The control contained no NaF or vanadate in the medium. Since the phosphatase inhibitory effect of these drugs is not evident at high ATP concentration, an additional assay (4 mM ATP) was carried out.

The presence of okadaic acid and particularly cyclosporin A in the pre-incubation of synaptosomal membranes at low ATP concentration inhibited insulin-stimulated PA synthesis from DPG and SAG. As expected, these actions were not observed at high ATP concentration. However, NaF strongly increased PA synthesis at low (Figure 5a) and high (Figure 5b) ATP concentrations. It can therefore be hypothesized that the stimulatory effect of NaF on PA synthesis is not related to Ser/Thr protein phosphorylation protection.

It is well known that the phosphorylation of inositol phospholipids phosphorylated in the 3 hydroxyl position by phosphatidylinositol 3 kinase (PI3K) is stimulated by insulin in a broad spectrum of insulin-responsive tissues (17). We speculate that NaF is also involved in a selective protein phosphatase acting on Akt Ser/Thr that could be phosphorylated by this transduction mechanism upstream of DAGK activation. However, in the presence of LY294002, a selective PI3K inhibitor, insulin stimulation of PA synthesized from DPG or SAG was unchanged (data not shown).

NaF and GTPγS activate PA synthesis from exogenous DPG or SAG

Many of the cellular activities of NaF are attributed to a fluoride complex with aluminium, forming fluoraluminates that activate heterotrimeric G proteins (18). In order to explore G protein participation in DAGK activity stimulated by insulin, pre-incubation with GTPγS, a full G protein activator (19) was evaluated. NaF and GTPγS activity was assessed as a function of time in the absence and the presence of the hormone. As shown in (Figure 6, left panels),

at 5 min incubation without the hormone, NaF and GTPγS activate PA synthesis from exogenous DPG, representing 306 % and 140 % of control values, respectively. When SAG is the substrate, NaF and GTPγS give rise to a similar increase in PA formation. However, when NaF and GTPγS are added after 5 min exposure to insulin along 5 min incubation time they fail to enhance PA synthesis (Figure 6, right panels).

PIP2 labeling was inhibited by GTPγS or NaF in the presence of exogenous DPG

G protein activation by GTPγS actively increases PPI labeling through PIP2 hydrolysis activation and its subsequent resynthesis. In Figure 6 and Figure 7, it can be seen that in the presence of exogenous DPG, GTPγS and NaF increase PA labeling but decrease PIP2 labeling with respect to the controls (Figure 7, left panel). GTPγS and NaF also serve to increase PA labeling in the presence of SAG, but in this case PIP2 labeling remains the same or increases instead of decreasing (Figure 7, right panel). These data are in agreement with the hypothesized close relationship between 18:0-20:4 PA formation and PPI phosphorylation (5).

Taking into account that PIP2 is a potent inhibitor of DAGKε we speculate that regulation of PIP2 levels through phospholipases C activation could be involved in the stimulation of PA formation.

3.9. NaF inhibited insulin action on polyphosphoinositides phosphorylation

Figure 8 shows the effect of insulin on PA and PIP plus PIP2 labeling in the absence and presence of NaF, with a sharp increase in PA formation when SAG is the substrate (77 % with respect to the control). The same effect was observed in the DPG assay with a 72 % PA synthesis compared to controls. This Figure also shows that NaF significantly increases PA formation with

respect to the insulin condition (52 % and 95 % in the presence of SAG or DPG, respectively). Insulin strongly increases PIP plus PIP2 labeling. However, when NaF is added to insulin, a strong inhibition of PIP plus PIP2 labeling was observed, showing values 80% lower with both DAG than in the insulin condition.

3.10. Insulin preferentially increased SAG phosphorylation from Hp membranes

Comparative experiments with Hp synaptosomal preparations were also carried out and revealed that PA synthesized from SAG or DPG is 18.16 or 3.58 times higher than from endogenous DAG, respectively (Table II). Insulin strongly increased DAGK action on endogenous DAG in synaptic terminals from Hp (199 % with respect to control). Insulin preferentially increased polyunsaturated PA synthesis from Hp membranes (175 % in Hp membranes and 65 % in CC membranes).

4. Discussion

PA formation is stimulated by insulin in CC synaptosomes from adult rats (5). Our previous results indicated that DAGK acts partly in response to increased DAG generated by insulin-activated PIP2-PLC and PLD-PAP2 pathways. This action is reinforced by an additional insulin-induced DAGK activation mechanism apparently related to polyunsaturated DAG phosphorylation. This effect was also observed when exogenous radioactive DAG ([3H]-DAG) of complex composition was used as substrate for DAGK determination (4).

In the present study it was demonstrated that membrane preparations from CC synaptic terminals preferentially synthesize PA from SAG rather than DPG. Although the use of polyunsaturated DAG was higher in the OG assay than in the DMSO assay, the preferential synthesis of polyunsaturated PA was

similar in the two cases. This observed preference for SAG was independent of the DPG (250 μM) or ATP (0.5 mM) concentration, since these values are not saturating substrate / co-substrate concentrations, respectively (data not shown). The possibility that unsaturated DAG acts as a positive regulator of a non acyl-selective DAGK was also discarded (Figure 1). Interestingly, PA synthesis from SAG was also found to be predominant in synaptosomes from Hp (Table II).

Ten mammalian DAGK isozymes have been identified to date, including the recently cloned DAGKκ (20). One of the important features of the DAGK family is that most of the isozymes, except for class II, are highly expressed in the brain, indicating their physiological importance in the central nervous system. DAGKε is unique among the DAG kinases in that it preferentially phosphorylates DAG with an arachidonate— an unsaturated fatty acid—in the sn2 position. This selectivity suggests that DAGKε has a more prominent role than other DAGK isoforms in enriching a precursor pool for inositol phospholipid synthesis with unsaturated fatty acids (8).

Interestingly, DAGKε-deficient mice had significantly shorter seizures following electroconvulsive shock and they also recovered faster than wild-type mice (21). In this DAGKε-deficient experimental model, examination of brain lipids revealed reduced levels of arachidonate in both PIP2 and DAG. Like human DAGKε, the murine counterpart expressed in COS cells displayed high selectivity for 20:4-DAG as compared with oleoyl-DAG (10-fold higher) (21).

The present study reports evidence of synaptosomal PA formation in a DAGK assay showing detergent (OG) and substrate (SAG) preference as well as insensitivity to R59022 and R59949 (DAGK inhibitors) in the absence and presence of calcium ions. All these data indirectly indicate the preferential formation of PA through a DAGKε activity under our assay conditions.

Our data also reveal that in a tyrosine phosphorylation-dependent mechanism, the level of the insulin-induced increase in PA synthesis is the same whether saturated or unsaturated exogenous DAG is used as substrate. As a consequence of the high preferential SAG utilization, unsaturated PA formation from CC synaptic terminals in the presence of insulin was significantly higher than saturated PA. On the other hand, insulin effect on PA formation occurs preferentially in SAG in Hp membranes, rather than CC.

The stimulatory effect on PA formation was reinforced by the simultaneous presence of insulin and NaF. Although NaF is a Ser/Thr phosphatase inhibitor (16) the mediation of PP1, PP2A or PP2B in the insulin-dependent activation was discarded by using the selective protein phosphatase inhibitors okadaic acid and cyclosporin A.

NaF is also a potent, rapid, and reversible activator of the regulatory heterotrimeric GTP-binding protein in virtually all in vitro systems. It has been reported that the effect of NaF on these G proteins is the result of the formation of AlF4 from fluoride and trace amounts of aluminum, which can come from contaminated glassware (22).

One can speculate that the NaF effect is mediated by a G protein-dependent activation of PIP2-PLC. Although the increase in saturated and unsaturated PA formation was similar with respect to the control level, PIP2 phosphorylation decreased sharply (DPG, Fig. 7, right panel) or increased slightly (SAG, Fig. 7, left panel). It can therefore be hypothesized that PIP2 hydrolysis occurs and a PIP2 resynthesis mechanism takes place as 18:0-20:4 PA increases.

Upon evaluation of phosphoinositide phosphorylation (PIP plus PIP2) under the usual ATP phosphorylation assay condition of DAGK, labeling increased in the presence of insulin. However, PIP+PIP2 phosphorylation decreased in the presence of NaF with respect to values obtained with the

hormone (Figure 8). Taking into account that PIP and particularly PIP2 are potent inhibitors of DAGKε (9), NaF reinforcement of DAGK insulin-stimulation could be due to a dual fluoride action that negatively regulates PPIs levels as previously described in brain slices exposed to carbachol and GTPγS (23; 24). These authors reported strong PIP2 depletion as a consequence of phospholipase C activation, and the inhibition of PPI synthesis.

It can be surmised that in the presence of insulin, NaF causes maximal depletion of PPIs level. A G protein-dependent activation of PIP2-PLC (AlF4 as activator) together with a diminution of PPIs availability through phosphatidylinositol kinase inhibition may occur.

The co-activation mechanism by G protein activators (such as GTPγS or NaF) could conceivably have been experimentally started in the absence of IR activation, but in this case it would most likely take place on a molecular target implicated in IR signaling (such as a G protein-related target).

It is known that the α subunits (αq, α11, α14, and α16) of all four members of the Gq subfamily of heterotrimeric G proteins activate PLC-β isozymes but not PLC-γ1 or PLC-δ1 (25). Polypeptide growth factors such as platelet-derived (PDGF), epidermal, fibroblast, nerve and hepatocyte growth factors all induce PIP2 turnover by activating PLC-γ in a wide variety of cells. Binding of these growth factors to their receptors results in the activation of intrinsic protein tyrosine kinase receptor activity and the consequent tyrosine phosphorylation of numerous proteins, including the receptor itself and PLC-γ (26). Thus, PLC-γ appears to be a good candidate to explain PIP2-PLC activation by insulin (5).

Our present results also lead us to suggest that increased PIP2 hydrolysis selectively stimulate the DAGKε isoform. This isoform could be negatively regulated when high PIP2 levels occur. How do we explain GTPγS

or NaF activation of this regulatory mechanism? We postulate an IR-related co-activation mechanism inducing stimulation of PIP2 resynthesis.

Using insulin receptors isolated from plasma membranes of human fat cells, it has recently been demonstrated that the activation of the insulin receptor autophosphorylation by insulin is sensitive to activators (e.g. GTPγS) as well as to inhibitors (e.g. GDPβS and pertussis toxin) of G-protein function (27). These authors also report that activation of G-proteins by GTPγS induces a 5-fold increase in receptor autophosphorylation over baseline values and a 3-fold increase in insulin-induced autophosphorylation.

Conversely it was reported that protein tyrosine phosphatases (PTPs) are essential for Gq/11 protein activation (28), which together with previous observations suggests that tyrosine kinases and PTPs co-operate to activate the Gq/11 protein by regulating tyrosine phosphorylation of the Gαq/11 subunit (28; 29).

How the insulin-related DAGK activation mechanism in CC synaptosomes is regulated by G protein efectors still remains to be elucidated.

It is known that phosphatidylinositols are enriched at the sn2 position with arachidonate. As previously mentioned, most DAGK isoforms do not distinguish between the fatty acid components of DAG *in vitro*, thus suggesting that *in vivo* phosphatidylinositols maintain their unsaturated fatty acid enrichment by coupling PI-specific phospholipase C enzymes with DAG kinases and other enzymes involved in resynthesizing PIP2. Coupling these enzymes could maintain the fatty acid components of PIP2 (30; 31).

DAGKε predominantly phosphorylates DAG with an arachidonate in the sn2 position (6), supporting the notion that DAGKε plays a more prominent role than other DAGK isoforms in enriching brain inositol phospholipids with

unsaturated fatty acids (8; 21). Although DAGKε is able to phosphorylate DPG or other saturated DAGs, it is highly efficient in the 2-arachidonoyl DAGs phosphorylation (32). It could therefore be suggested that 2-arachidonoyl PA is involved in the synthesis of phosphatidylinositol and the replenishment of the precursor by phosphorylations to PIP and PIP2 or as a phosphoinositide kinase regulator. Further studies will be necessary to explain this in the context of neural insulin signaling.

Insulin and its receptor are widely localized throughout the brain though most densely found in the olfactory bulb, cerebral cortex, hypothalamus, and hippocampus. They are related to various functions including regulation of glucose metabolism, food intake and body weight, learning, memory, and attention (3; 33; 34). At the synapse, insulin receptors regulate neurotransmitter release and receptor recruitment, indicating a potential involvement of insulin in synaptic plasticity (1). However, the molecular mechanism by which insulin exerts these beneficial effects has not been elucidated to date.

The present results suggest that DAGKε activity is stimulated by insulin in synaptic terminals from Hp and CC and that insulin preferentially stimulates PA formation from SAG in Hp synaptosomes. The stimulation of IR-related PIP2 hydrolysis as we previously reported (5) could remove PIP2 as a DAGKε-negative regulator. Insulin-induced DAGKε activity could participate in a final PIP2 resynthesis mechanism as a consequence of G protein-mediated PIP2-PLC activation. In conclusion, activation of DAGKε and arachidonate-rich PA formation may potentially be involved in inositol lipid homeostasis at the synapse, opening an interesting avenue for brain insulin signaling studies.

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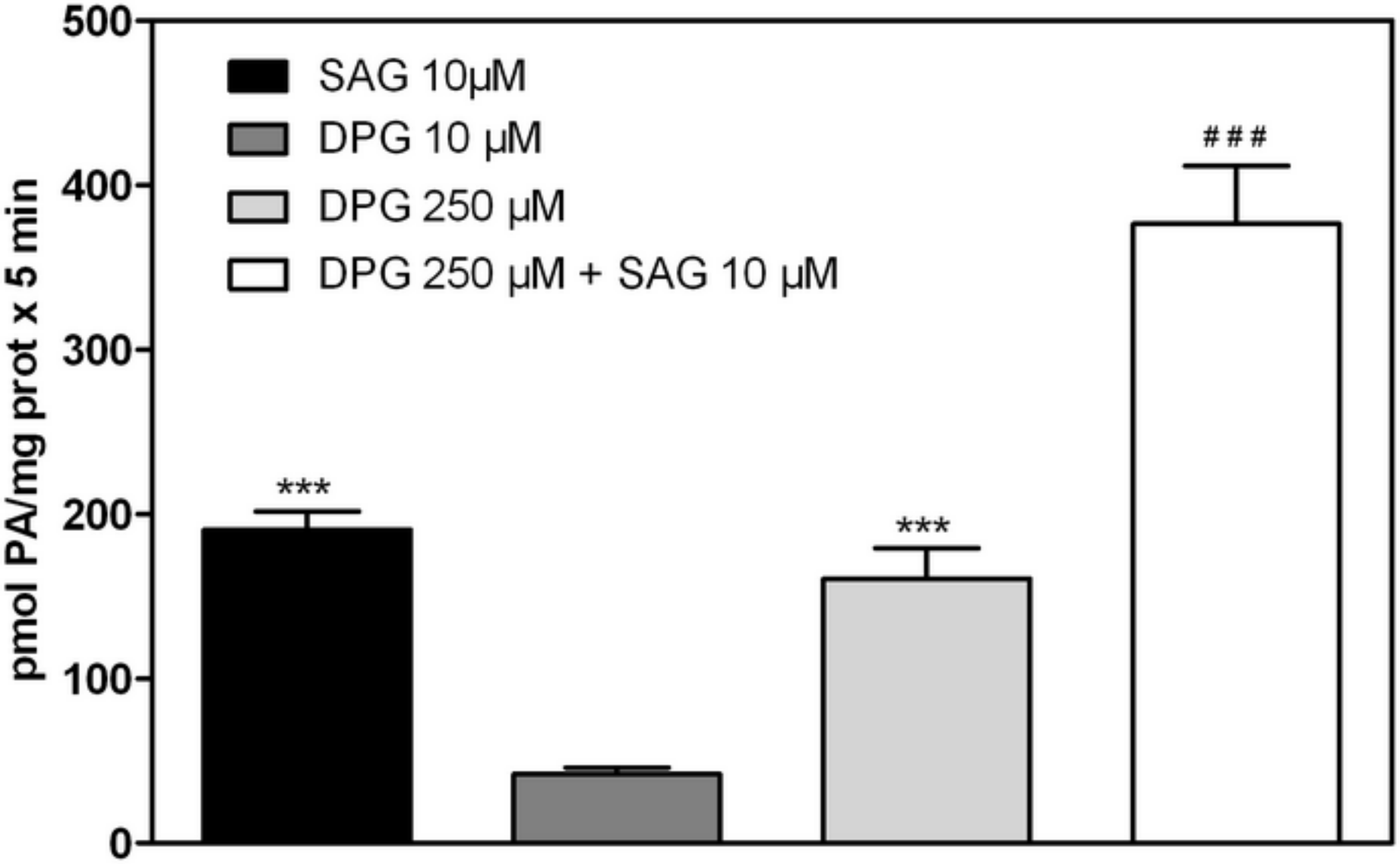

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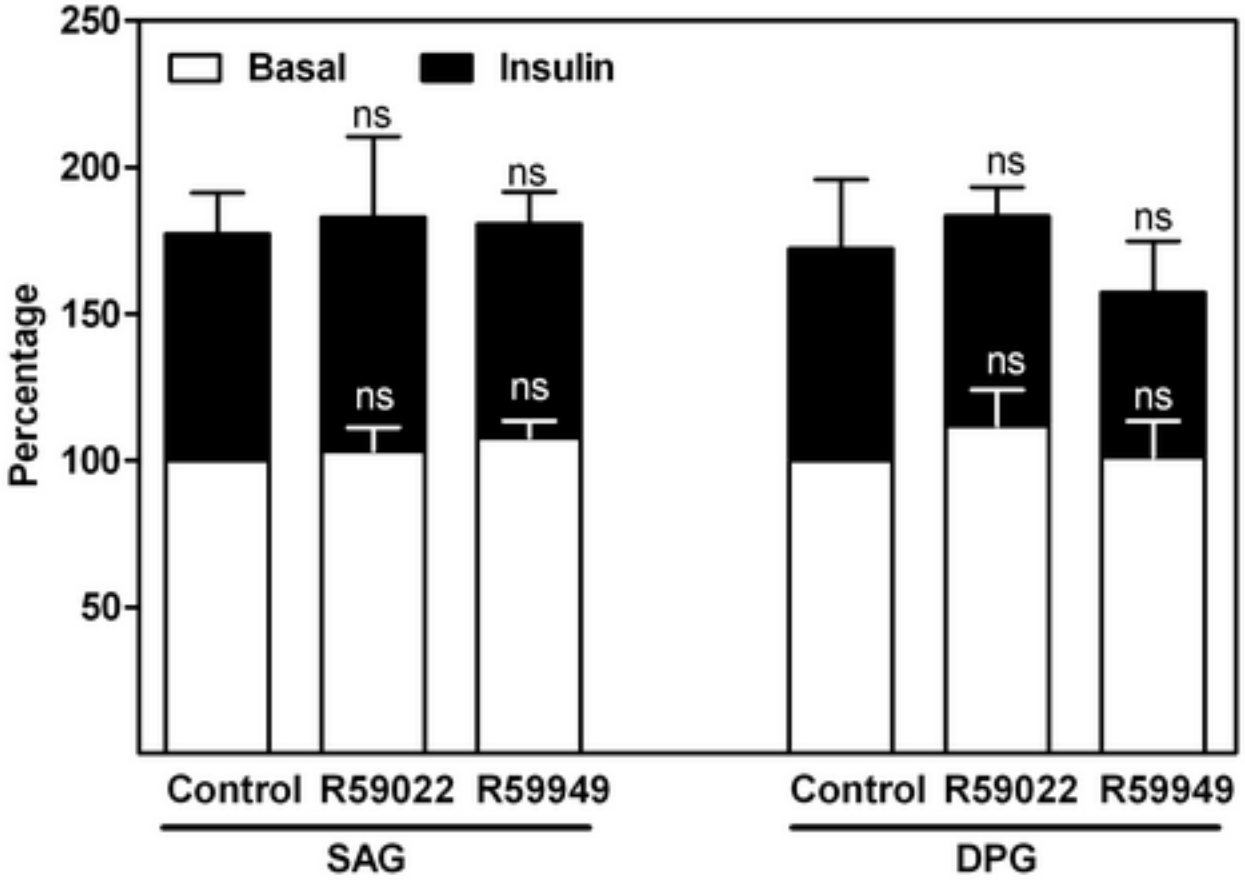

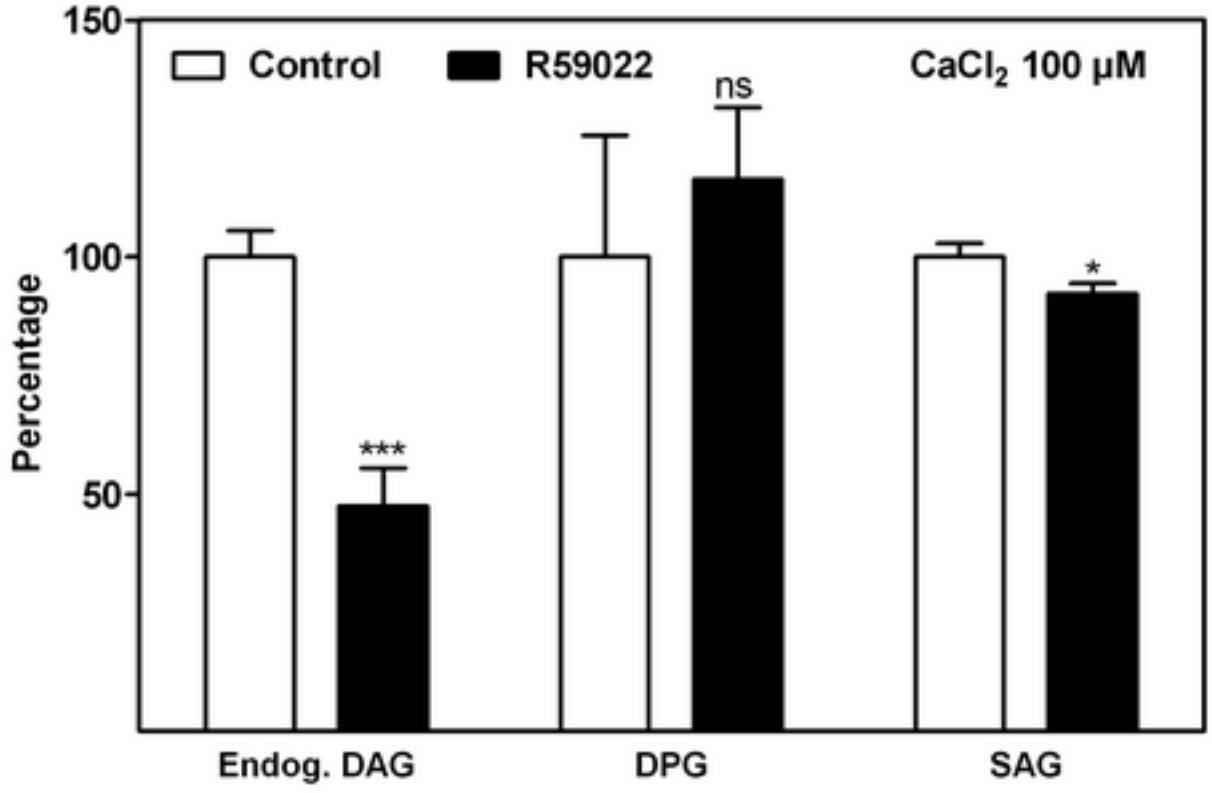

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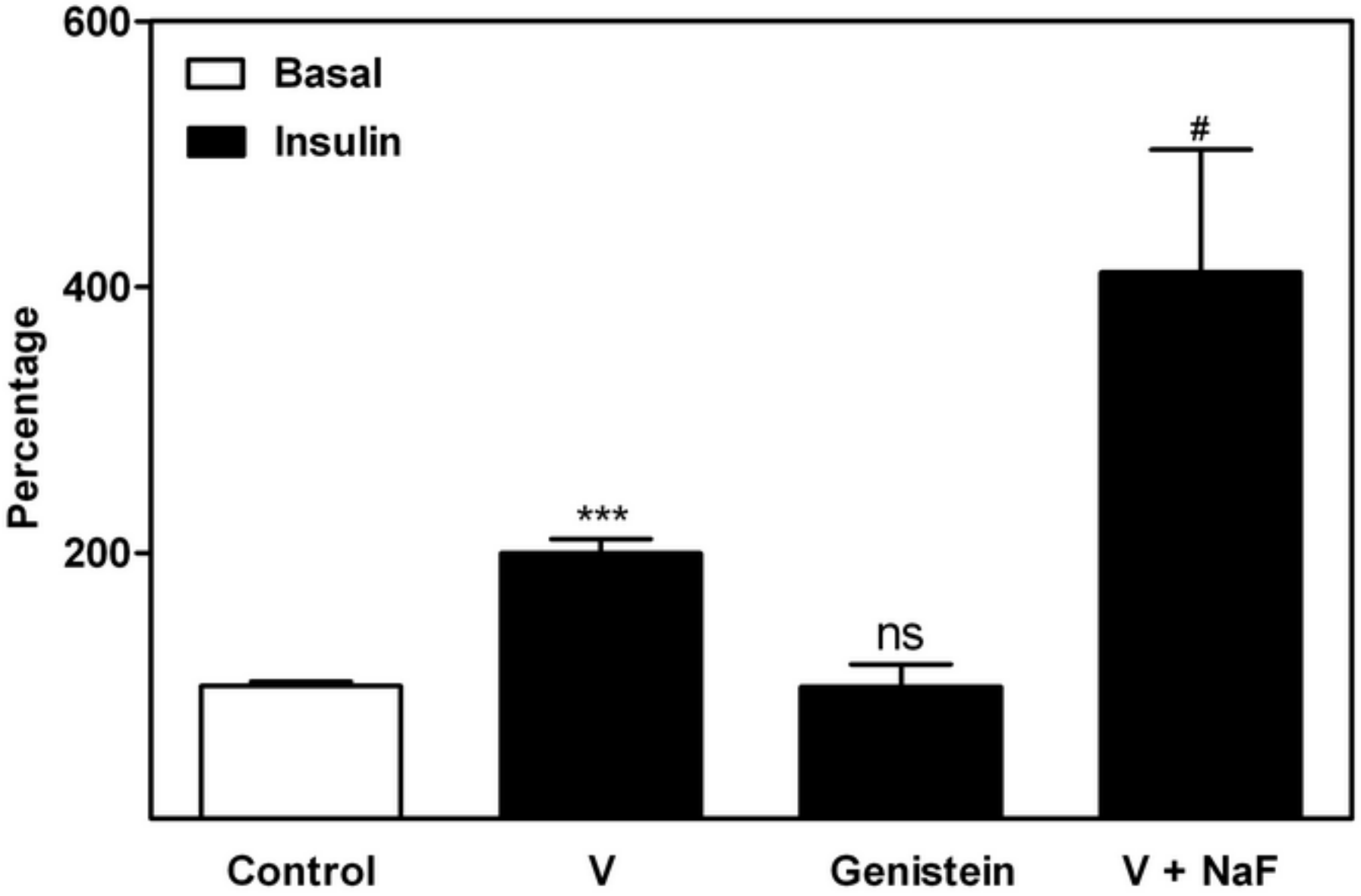

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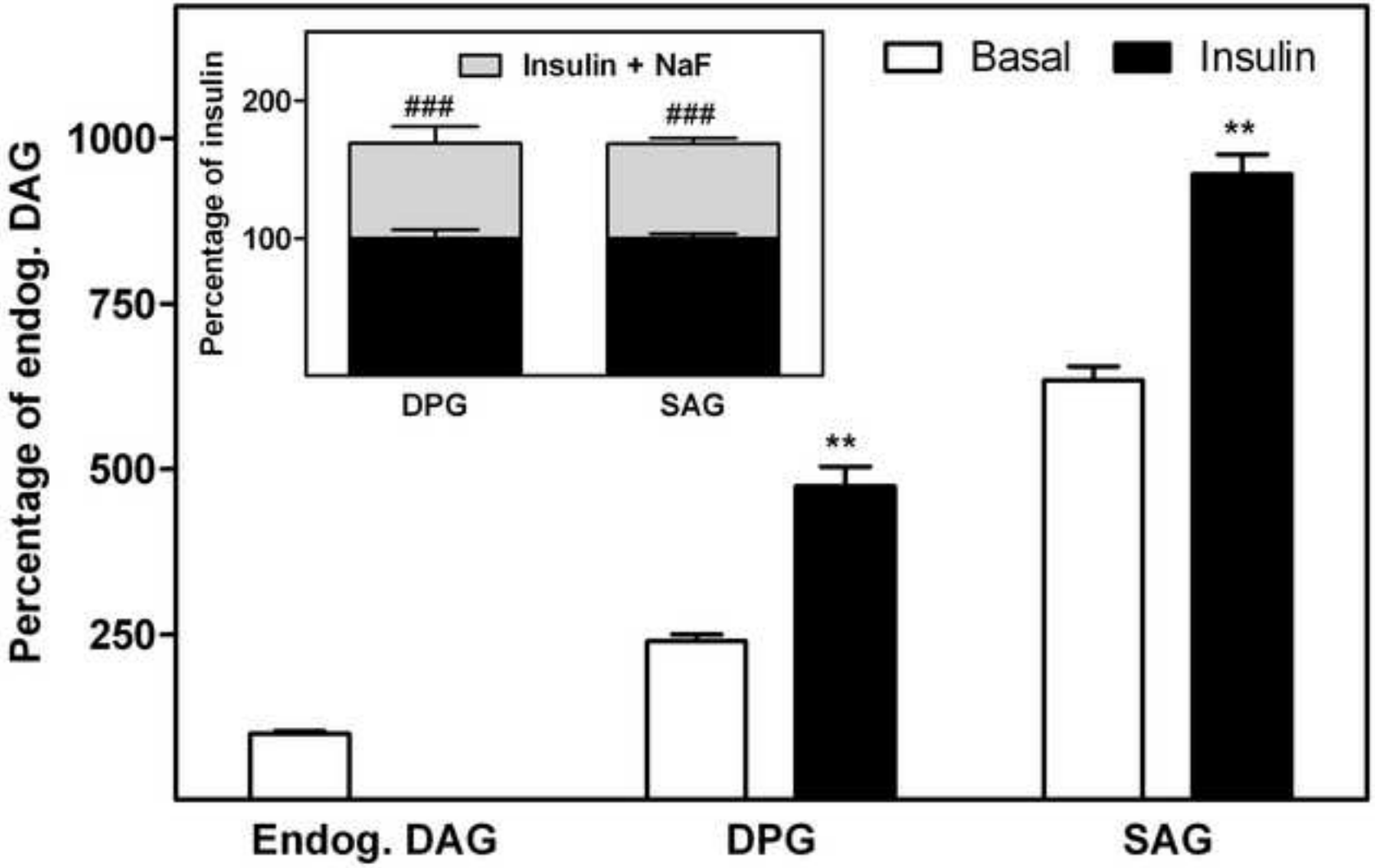

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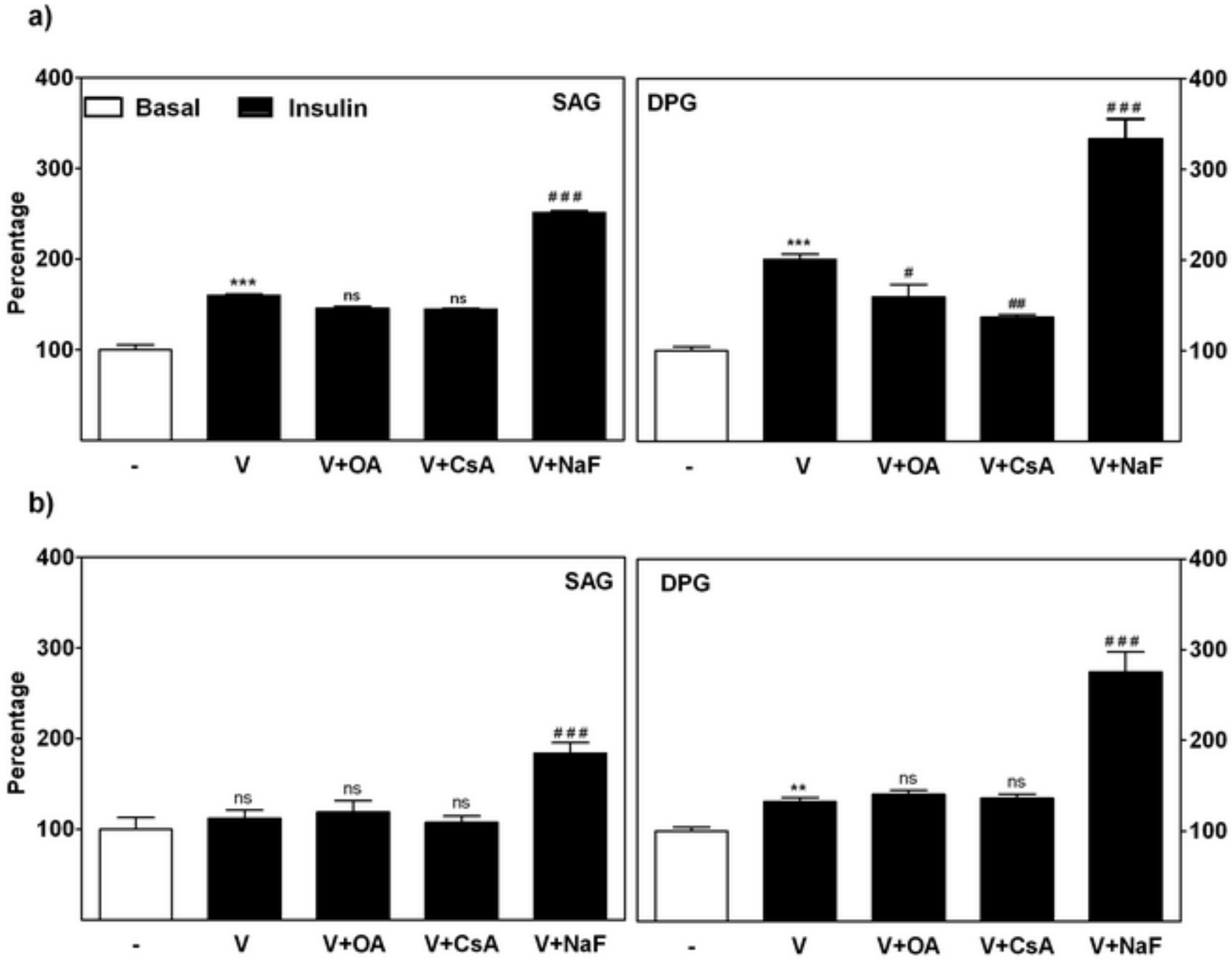

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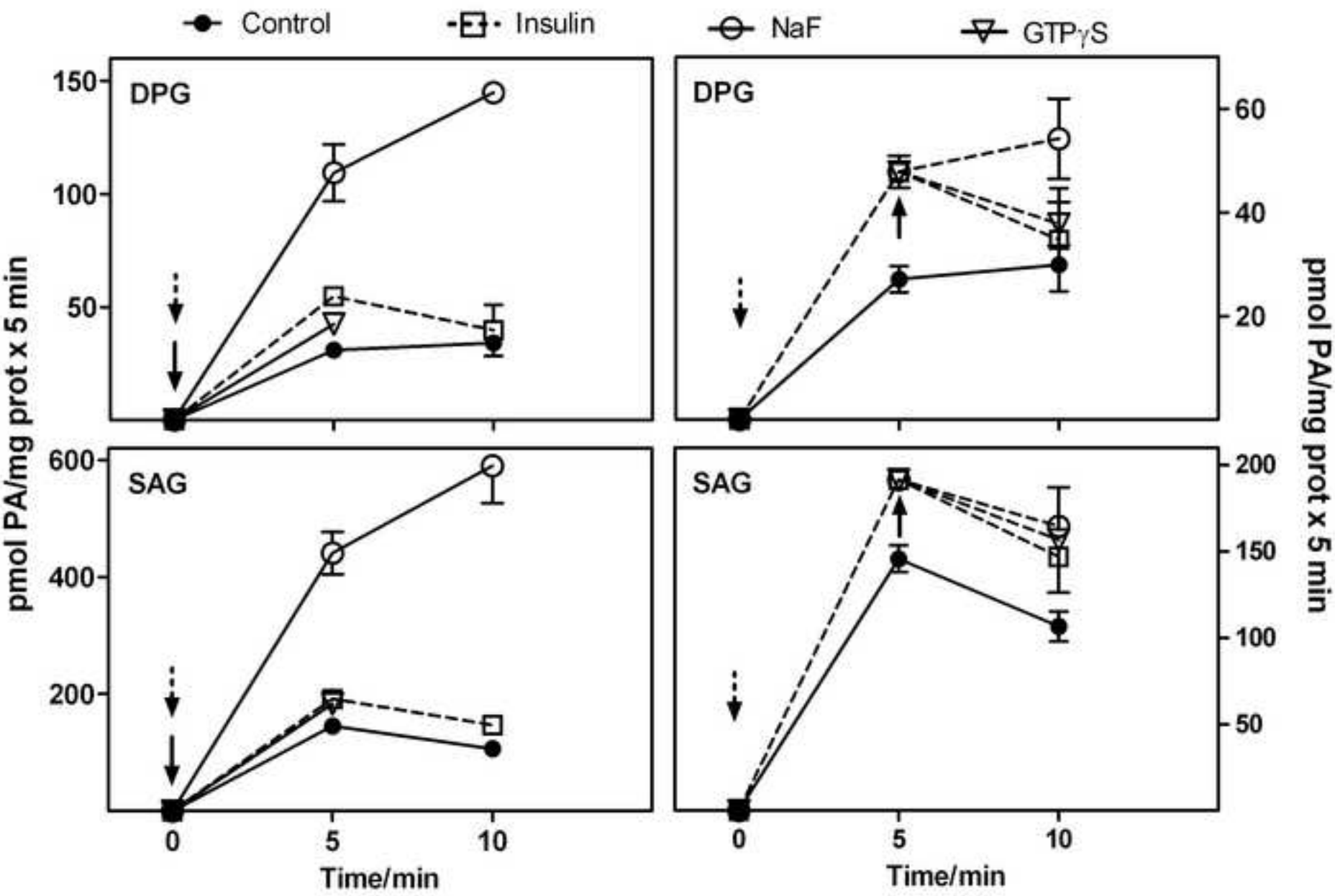

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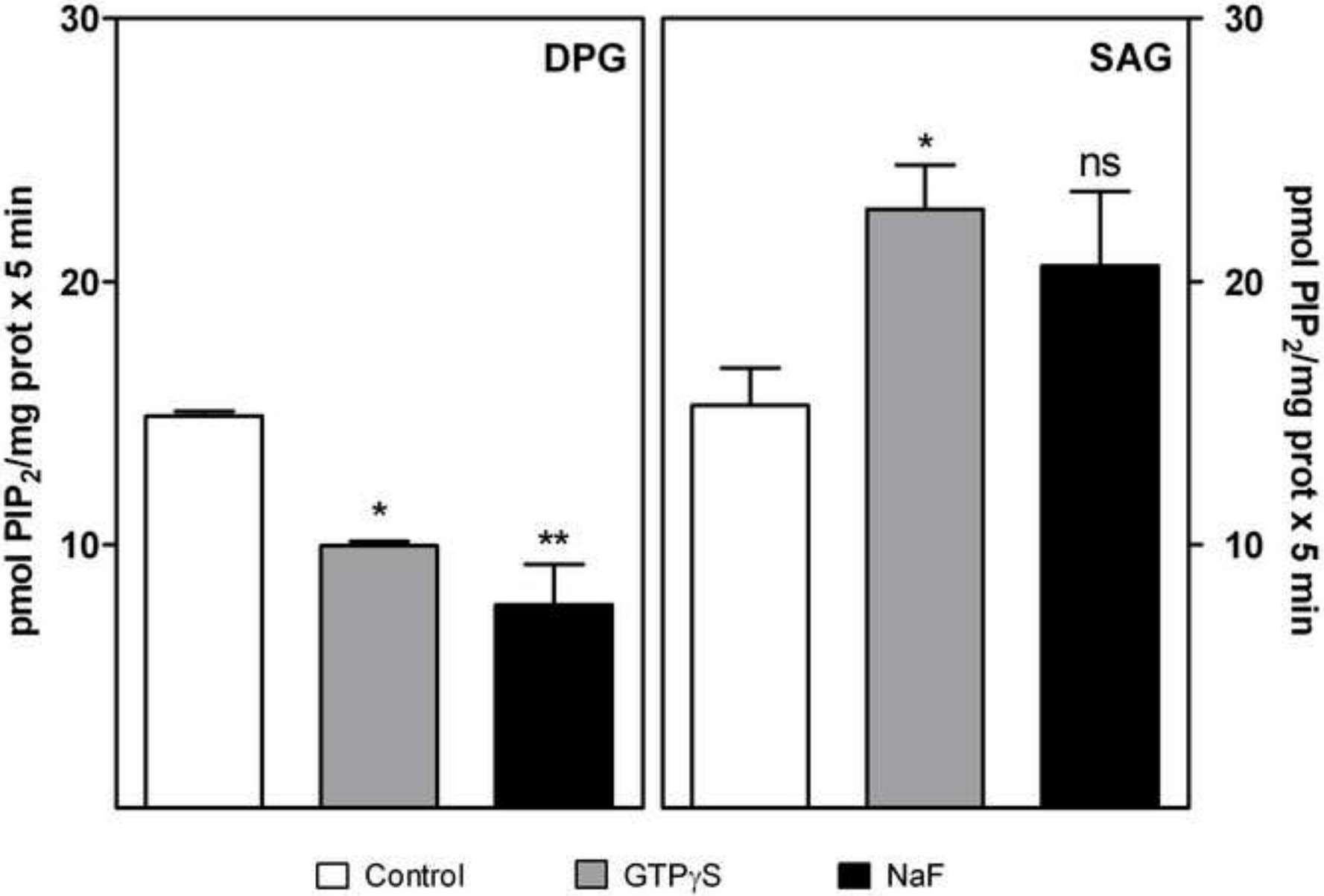

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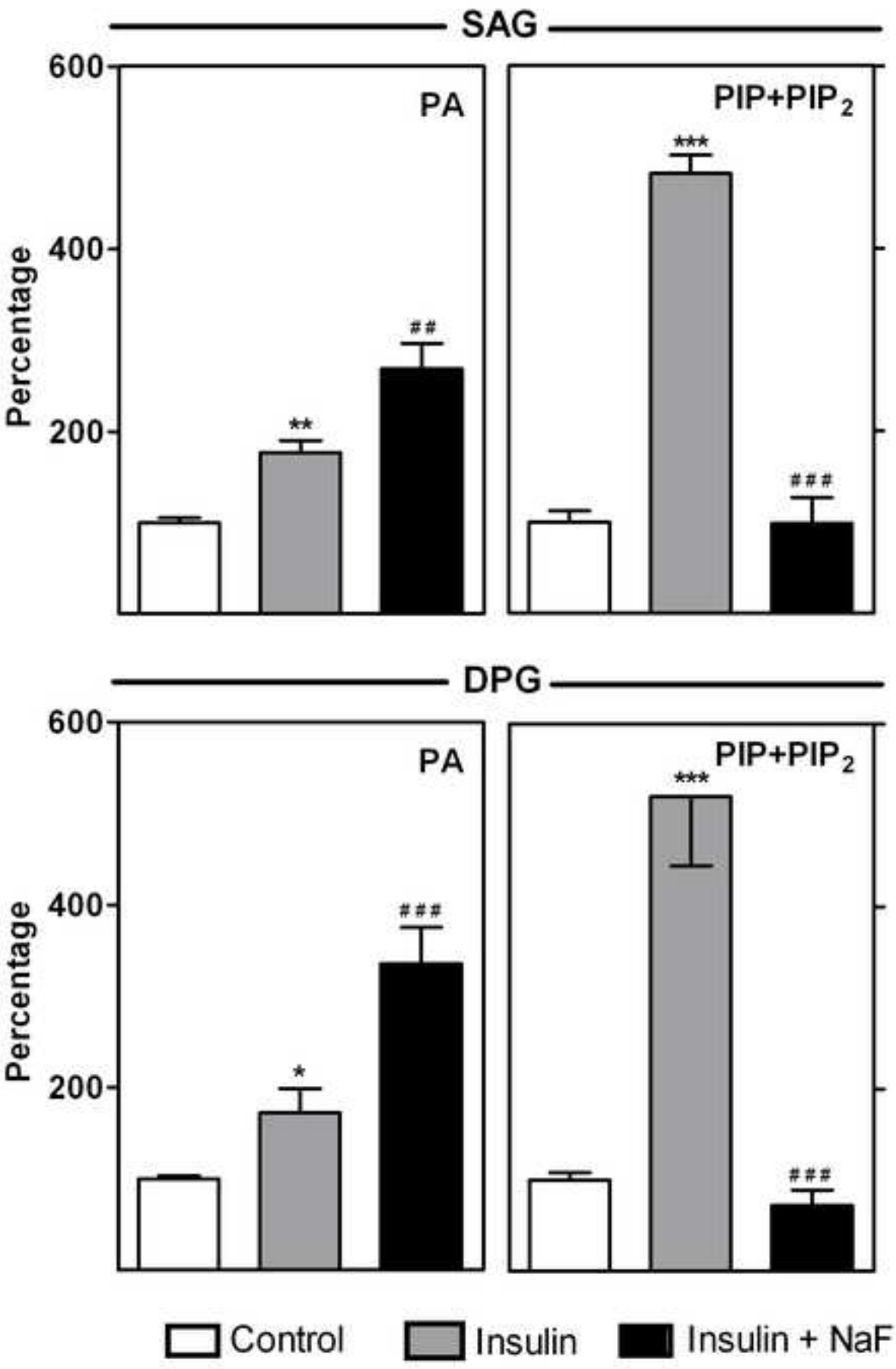

Fig. 1: Is SAG a positive regulator of DPG phosphorylation?

DAGK activity was determined in synaptosomal membranes incubated during 5 min in the assay condition as described under "Experimental Procedures" by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate. The lipid substrate was 250 μM 1,2-dipalmitoyl-sn-glycerol (DPG) either alone or in combination with 10 μM 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) in a detergent-micellar assay (OG as detergent). Controls have 10 μM DPG or SAG. Lipids were resuspended in dimethylsulfoxide (at a final concentration of 0.1 %). Results are expressed as the mean ± S.D. of the incorporation values obtained in three individual samples.

***P < 0.0001 vs. DPG 10 μM, $^{###}$P < 0.0010 vs. DPG 250 μM.

Fig. 2: DAGK activity with SAG or DPG in the presence of insulin. R59022 and R59949 effects.

Upper panel: DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition as described under "Experimental Procedures" by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the presence of 250 μM SAG or DPG in the absence or presence of 200 nM insulin. Five minutes of incubation was carried out after ten minutes of pre-incubation in the absence or in the presence of either 10 μM R59022 or 0.24 μM R59949. In all insulin conditions 200 μM vanadate was present. The lipid was resuspended in DMSO at a final concentration of 0.1% in the assay condition without exogenous calcium ions. **Lower panel:** DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition described under "Experimental Procedures", modified by the presence of 0.1 mM calcium ions,

by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the presence of endogenous DAG or with either 250 µM SAG or DPG (DMSO as detergent). Incubation for five minutes after ten minutes of pre-incubation in the absence or presence of 10 µM R59022 was carried out. Lipids were resuspended in DMSO (at a final concentration of 0.1 %). Results are calculated as percentages of incorporation values. Controls are set at 100 and the values are means ± S.D. of six individual samples. In upper panel, the significance degrees were calculated vis-à-vis their respective controls (basal or insulin) without R59022 and R59949. In the lower panel, the significance levels were calculated vis-à-vis their respective controls without R59022.

*P < 0.050, ***P < 0.0010.

Fig. 3: Insulin effect on synaptosomal DAGK action on exogenous SAG measured with either genistein or vanadate. Effect of sodium fluoride. DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition as described under “Experimental Procedures” by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the presence of 250 µM SAG (DMSO as detergent). The incubation time was started with insulin addition after ten min pre-incubation in the presence of inhibitors. Results are calculated as percentages of incorporation values. Controls are set at 100 and the values are means ± S.D. of six individual samples. Significance levels were calculated vis-à-vis the controls.

***P < 0.0001 vs. control, $^{\#}$P < 0.050 vs. insulin condition.

Fig. 4: Effect of insulin on synaptosomal DAGK action on DPG and SAG. Sodium fluoride action.

DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition as described under “Experimental Procedures” by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the absence (endogenous DAG) or in the presence of either 250 µM DPG or SAG (DMSO as detergent). The incubation time was started with insulin (200 nM) addition after ten min pre-incubation in the presence of vanadate (200 µM) (black bars, insulin condition) or insulin plus NaF (insert, stacked gray bars). Results are calculated as percentages of incorporation values. Control (endogenous DAG as lipid substrate) is set at 100 and the values are the mean ± S.D. of six individual samples. Significance levels were calculated vis-à-vis the control condition. **Insert**: NaF action on synaptosomal DAGK activity in the presence of insulin (DPG or SAG as substrates) is calculated as a percentage of mean incorporation values from insulin condition (set at 100). The significance levels of insulin conditions were calculated vis-à-vis the respective basal condition. The significance levels of insulin+NaF conditions were calculated vis-à-vis the respective insulin condition.

**P < 0.005 vs. basal, $^{###}$P < 0.0001 vs. insulin condition.

Fig. 5: Effect of insulin on synaptosomal DAGK action on DPG and SAG. Action of Ser/Thr protein phosphatase inhibitors.

DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition as described under “Experimental Procedures” by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the presence of either 250 µM DPG or SAG (DMSO as detergent). Incubation time was started with insulin (black bars) addition after ten min pre-incubation in the absence (basal) or in the

presence of vanadate (V) or plus 1 µM okadaic acid (V+OA) or 120 nM cyclosporine A (V+CsA) or 20 mM NaF (V+NaF). Results are calculated as percentages of incorporation values. Controls are set at 100 and the values are the mean ± S.D. of six individual samples. The significance level of V condition was calculated vis-à-vis the basal condition. The significance levels of V+OA, V+CsA and V+NaF conditions were calculated vis-à-vis the V condition.

*P < 0.050, **P < 0.005, ***P < 0.0001 vs. control.

$^{\#}$P < 0.050, $^{\#\#}$P < 0.005, $^{\#\#\#}$P < 0.0005 vs. insulin condition.

Fig. 6: Synaptosomal DAGK action on DPG or SAG as substrate as a function of time. Effect of GTPγS and NaF in the absence and presence of insulin.

DAGK activity was determined in CC synaptosomal membranes incubated during 5 min in the assay condition as described under “Experimental Procedures” by measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate in the presence of either 250 µM DPG or SAG (DMSO as detergent). Incubation time was started at zero time. **Left panels**: 200 nM insulin (dashed arrow) or 100 µM GTPγS or 20 mM NaF (black arrow) were present from zero time incubation. **Right panels**: 100 µM GTPγS or 20 mM NaF (black arrow) were added after five min pre-incubation in the presence of insulin (dashed arrow). In all insulin conditions 200 µM vanadate was present. Results are expressed as the mean ± S.D. of the incorporation values obtained in three individual samples.

Fig. 7: Effect of GTPγS and NaF on synaptosomal phosphatidylinositol bis-phosphate (PIP2) labeling with [γ-32P]ATP in the presence of exogenous DPG or SAG.

PIP2 labeling was obtained measuring radioactive phosphate incorporation through [γ-32P]ATP as radioactive substrate in the presence of either 250 μM DPG or SAG (DMSO as detergent). GTPγS (100 μM) and 20 mM NaF were present from zero time and during 5 min incubation. Results are expressed as the mean ± S.D. of the incorporation values obtained in six individual samples. The significance levels of the GTPγS and NaF condition were calculated vis-à-vis the respective control condition.

***P < 0.0001.

Fig. 8: Effect of insulin on synaptosomal PA and PIP+PIP2 labeling with [γ-32P]ATP in the presence of exogenous DPG or SAG. Sodium fluoride action.

PA, PIP and PIP2 labeling was obtained measuring radioactive phosphate incorporation through [γ-32P]ATP as radioactive substrate during 10 min in the presence of either 250 μM DPG or SAG (DMSO as detergent). Insulin was added after ten min pre-incubation in the presence of vanadate or vanadate plus sodium fluoride. Results are calculated as percentages of incorporation values with respect to control condition. The significance levels of the insulin condition were calculated vis-à-vis the control. The significance levels of the insulin+NaF condition were calculated vis-à-vis the insulin condition.

*P < 0.050, **P < 0.0010, ***P < 0.0005 vs. control.

$^{\#\#}$P < 0.0010, $^{\#\#\#}$P < 0.0005 vs. I+V.

Table I
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Table I: DAGK activity from CC synaptosomes measured with either DPG or SAG in a detergent-lipid micellar assay.

DAGK activity was determined in CC synaptosomal membranes measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate and with either 1,2-dipalmitoyl-sn-glycerol (DPG) or 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) in a detergent-micellar assay. The lipid was resuspended in 50 mM octyl-β-glucopyranoside (OG) at final concentrations of 0.5 mol% in the assay condition as described under "Experimental Procedures". Results are expressed as the mean ± S.D. of the incorporation values obtained in three individual samples.

	OG Assay (50 mM)		DMSO Assay	
Conditions	**%**	**SD**	**%**	**SD**
Basal	100	8,88	100	3,05
DPG	341	38,74	209	35,71
SAG	1818	108,26	800	185,98

Table II
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Table II: **Insulin effect on DAGK activity from CC or Hp synaptosomes measured with either DPG or SAG in a detergent-lipid micellar assay.**

DAGK activity was determined in synaptosomal membranes incubated during 5 min in the assay condition as described under "Experimental Procedures". Synaptosomes were obtained from cerebral cortex (CC) or hyppocampus (Hp) and DAGK activity was determined measuring radioactive phosphate incorporation into PA through [γ-32P]ATP as radioactive substrate and with either 1,2-dipalmitoyl-sn-glycerol (DPG) or 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) in a detergent-micelar assay. The lipid was resuspended in 50 mM octyl-β-glucopyranoside (OG) at final concentration of 250 μM. Insulin (200 nM) plus vanadate (200 μM), as insulin condition, were present from zero time. Results are expressed as the mean ± S.D. of the incorporation values obtained in six individual samples.

Tissue	Lipid Substrate	Control (pmole PA/mg prot x min)	Insulin (pmole PA/mg prot x min)
Hippocampus	Endog. DAG	0.737 ± 0.018	2.202 ± 0.134
	SAG	14.123 ± 0.708	38.893 ± 2.366
	DPG	3.377 ± 0.394	8.865 ± 1.133
Cerebral Cortex	Endog. DAG	0.743 ± 0.066	1.467 ± 0.196
	SAG	13.506 ± 0.710	22.228 ± 2.494
	DPG	2.531 ± 0.445	6.300 ± 0.509